DESCRIPTION

VACCINE PREPARATIONS CONTAINING ATTENUATED TOXIN

5   Technical Field

The present invention relates to vaccine preparations that contain an attenuated toxin as the adjuvant and that are useful for treating and preventing diseases.

10   Background Art

Vaccines have been used to prevent various diseases, and tremendous results have been obtained. Nonetheless, vaccines also have side effects and there are many cases in which vaccines are not very effective. Thus, there is a strong need for improved vaccines.

15   Currently, many types of vaccines, for human or other animals, use pathogens or parts thereof as antigenic materials for vaccines. Thus, there is no denying the possibility that vaccines may be contaminated with constituents of pathogens or ingredients of the growth medium for the pathogens. These contaminants can provoke

20   undesirable side reactions in vaccination. In addition, antigenic domains themselves, pertaining to immunity, can induce side reactions when inoculated in large quantity.

In an effort to avoid such side reactions as much as possible and manufacture vaccines excellent in safety, those in the art have

25   reduced the inoculum size of vaccine antigen, improved the purity of antigen for vaccination, and/or administered a vaccine by a route other than injection. However, generally such revision results in the reduction of immunogenic activity of the vaccine. Previously, adjuvants have been used as an effective countermeasure to this problem.

30   However, there still remain some problems to be solved, such as improving the effectiveness and safety of the adjuvant.

So far most vaccine inoculations are given by injection. This results in elevation of antibody titer in the blood. When the titer is maintained at a high level, it inhibits the propagation of pathogenic

35   microorganisms and thereby preventing diseases associated with the organisms. On the other hand, various viruses, such as influenza virus,

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as well as bacteria infect *via* mucous membranes of the respiratory tract. To prevent such diseases at an early stage of infection, vaccines capable of significantly enhancing local immunity, in the mucous membrane rather than in the blood, are preferred. To achieve

5   this goal, excellent adjuvants that help potentiate local immunity are desired. In other words, the development of an excellent adjuvant, that is effective and safe and that matches the type of antigen and vaccination route to be used, is an important subject in the development of new and improved vaccines.

10   Noteworthy inoculation routes other than injection include oral, percutaneous and intranasal administration. Injections must be conducted by medical staffs. Therefore, the vaccine inoculation by injection becomes problematic, for example, when it is necessary to vaccinate many people under a condition with poor medical facilities.

15   On the other hand, oral, percutaneous or intranasal administration can be performed with the instruction of a medical professional but without direct assistance as far as vaccine preparations are available. However, in general, sufficient immunological stimulation is hardly attainable with these routes of administration, and therefore suitable

20   adjuvants for such routes are demanded.

    Previously, aluminum compounds (aluminum sulfate, aluminum hydroxide, aluminum phosphate, etc.) have been widely used as adjuvants for vaccines. Currently, the gel of aluminum compound is almost the only adjuvant available for vaccines of human use. However, there

25   are some problems in regard to the aluminum adjuvant, and thus improvements are needed. Illustrative problems are pointed out as followings:

1) Problems associated with the production of aluminum adjuvant as well as its handling are as follows: since the quality of aluminum

30   adjuvant tends to vary from one production lot to another, it is not suited to large scale manufacturing. Moreover, the compound tends to be interfering in column chromatography of co-existing proteins, and is therefore inconvenient to handle.

2) Another problem associated with the aluminum adjuvant is that while

35   it excels in potentiating the humoral immunity, the compound is less effective for potentiating cellular immunity. Thus the types of

antigens to be used together are limited.

Research and development of new types of adjuvants, such as bacterial toxins, that overcome the drawbacks discussed above are in progress. Some illustrative examples are as follows:

5　1. Bacterial toxins, such as cholera toxin, and the like;

2. Substances having detergent activity, such as saponins, higher fatty acids, and the like;

3. Constituents of microorganisms or plants, such as BCG, muramyl peptide, and the like;

10　4. Functional proteins including cytokines, such as interleukin, heat shock protein, and the like;

5. Synthetic polyanion, polycation, and the like; and

6. Micro-carriers and the like;.

It is well known that certain bacterial toxins have adjuvant
15　activity, namely, activity of enhancing production of antigen-specific antibody. Among them, cholera toxin exhibits relatively high adjuvant activity (J. Holmgren et al., Vaccine 11, 1179-1184, 1993). The present inventors have been studying and developing vaccines containing adjuvants such as cholera toxin, *E. coli* heat-labile toxin, and the
20　subunits thereof (Unexamined Published Japanese Patent Application (JP-A) No. Hei 2-243633).

In addition to these studies, the ongoing research and development, which relates to vaccines containing cholera toxin, *E. coli* heat-labile toxin, pertussis toxin, and such as the adjuvant,
25　includes the following examples: influenza vaccine (K. Komase et al., Vaccine 16, 248-254, 1998; A. S. Tamura et al., Eur. J. Immunol. 21, 1337-1344, 1991), herpes simplex virus vaccine (C. M. Richards et al., Vaccine 15, 1065-1069, 1997), HIV-I vaccine (I. M. Belyakov et al. Proc. Natl. Acad. Sci. USA 95, 1709-1714, 1998), *Helicobacter*
30　*pylori* vaccine (R. Weltzin et al., Vaccine 15, 370-378, 1997), toxoplasma vaccine (I. Bourgiun et al., FEMS Immunol. Med. Microbiol 12, 121-126, 1995), measles vaccine (C. D. Partidos et al., Imunology 89, 483-487, 1996), poliovirus vaccine, etc.

However, the usage of vaccines added with bacterial toxins as
35　adjuvant in clinical practice might be of problem, because bacterial toxins are originally toxic to humans. The present inventors assumed

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that the toxins can be used safely for vaccines because the effective concentration of adjuvant toxins is sufficiently below the level where the toxins exhibit toxicity to humans and other animals. Thus, the present inventors studied the possible problems associated with

5   repeated administration of the adjuvanted intranasal vaccine and reported the result previously (S. Tamura et al., Vaccine 15, 1784-1790, 1997). Nonetheless, many experts have expressed concerns about safety of toxin-adjuvant vaccine due to, for example, the difference of susceptibility to cholera toxin between human and experimental animals

10 as well as the possibility of causing diarrhea (M.M. Levine et al. Microb. Rev. 47, 510-550, 1983).

The following methods have been proposed in order to take advantage of excellent activity of bacterial toxin in enhancing immunity and simultaneously to solve the problem of safety. However,

15 none of the methods come close to reaching the goal of realizing both safety and adjuvant activity.

(1) Attenuation by chemical or physical treatment:

A method to reduce the activity of toxin without losing the adjuvant activity — wherein the toxin is treated with formalin,

20 glutaraldehyde, acid, alkali, etc. or treated at a temperature severe for the toxin. For example, it is known that cholera toxin treated with glutaraldehyde has a toxic activity reduced to about 1/1000 as compared with the natural one while maintaining adjuvant activity (X. Liang et al., J. Immunol. 143, 484-490, 1989). However, toxin

25 activity reduced to 1/1000 relative to that of the natural toxin is evaluated not to be sufficiently safe.

(2) Search for cross-reacting mutant proteins produced by mutant strains:

A method for searching cross-reacting mutant proteins of interest

30 which retain adjuvant activity — wherein toxin-producing bacteria are treated with a mutagenizing chemical agent and toxins produced by resulting mutant strains are searched to find proteins of interest. For example, a cross-reacting protein (cross-reacting material; CRM), which showed low toxic activity and was reactive to anti-pertussis

35 toxin antibody, was found in culture medium of a pertussis toxin-producing bacterial mutant strain. This mutant toxin is

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immunogenic and can be a candidate for the pertussis vaccine antigen
(Y. Sato et al., Dev. Biol. Stand. 73, 93-107, 1991). In addition
to this, there is a possibility that the mutant toxin might be used
as the adjuvant with low toxic activity. However, the degree of toxic
5   activity and adjuvant activity of the mutant toxin has not been reported.
      Cross-reacting protein of diphtheria toxin, CRM-197 (DT-G52E)
(T. Uchida et al., J. Biol. Chem. 248, 3838-3844, 1973; G. Giannini
et al., Nucleic Acids Res. 12, 4063-4069, 1984), and a cross-reacting
protein of pseudomonad enterotoxin A, CRM-66 (H462Y) (S.J. Cryz et
10   al., Rev. Infec. Dis. 5 Suppl. 5, S992-S997, 1983; S. P. Kessler et
al., J. Biol. Chem. 267, 19107-19111, 1992) were reported as mutant
toxins which substantially lack ADP-ribosyltransferase activity. In
these mutant toxins, the ADP-ribosyltransferase activity was used
as the only marker for the toxic activity of these mutant toxins,
15   and thus there is no denying of the existence of residual toxic activity
generated by other mechanisms. Thus, it is possible that these mutants
have other toxin activities. In addition, it was reported that when
chemically linked to a polysaccharide antigen, although having
adjuvant activity, CRM-197 (DT-G52E) did not stimulate sufficient
20   antibody titers, and that consequently it required the use of additional
second adjuvant (G. S. Bixler et al., Adv. Exp. Med. Biol. 303, 185-190,
1991; D. M. Granoff et al., Vaccine 11 Suppl. 1, S46-S51, 1993).
      (3) Preparation of attenuated mutant toxins by genetic
engineering:
25   A method for constructing recombinant toxins, which still retain
adjuvant activity, - wherein the toxic activity is reduced by
artificially altering one or more amino acid residues of the toxin
molecule utilizing gene recombination techniques. As examples,
recombinants of E. coli heat-labile toxin, wherein the arginine residue
30   (Arg/R) at amino acid position 7 or 192 from the N-terminus was
substituted with a lysine residue (Lys/K) (LT-R7K, M.T. De Magistris
et al., Dev. Biol. Stand. 92, 123-126, 1998) or with glycine residue
(Gly/G) (LT-R192G, B. L. Dickinson et al., Infection Immun. 63,
1617-1623, 1995), were reported to substantially lack the toxic
35   activity but still retain the adjuvant activity.
      However, according to experiments of an other research group,

the toxic activity was not negligible in the above-mentioned recombinant toxins. Further, the recombinant proteins were unstable as compared with the natural one. As a result, larger amounts of the recombinant protein (relative to that of the natural one) must be

5   used to raise antibody titer to the same level as that given by the natural one (K. Komase et al., Vaccine 16, 248-254, 1998; C. C. R. Grant et al., Infect. Immun. 62, 4270-4278, 1994).

When a toxic activity is caused by multiple activities, the reduction of any one of the activities can result in the manifestation

10   of the other activities. The attenuation of toxin can be associated with this type of phenomenon, and therefore the marker of toxic activity should be selected carefully. As described above, attenuated adjuvants, which have been reported so far to exhibit very low toxicity, still have problems to be solved in regard to adjuvant activity, safety,

15   stability, and productivity. Further, many screening steps must be repeated to achieve reduction of toxic activity and preservation of adjuvant activity, which are contrary subjects, by amino acid substitution. Therefore, it is a time-consuming work.

20   Disclosure of the Invention

An object of the present invention is to provide an attenuated toxin, that has substantially no toxic activity but still has the excellent adjuvant activity of the toxin, as a new adjuvant.

Another object of the present invention is to provide a vaccine

25   using one or more adjuvants of the present invention, that is safe and effective and that can be obtained by relatively simple and convenient procedures. More specifically, the object is to provide a vaccine that can be used in reduced doses, or can be used without loss of its immunogenic activity when administered by a vaccination

30   route other than injection.

The present inventors have studied methods to reduce the toxic activity of a variety of toxins and found out that an attenuated toxin which can be prepared by a chemical treatment has substantially no toxic activity, but still has sufficient adjuvant activity of the

35   levels to meet the criteria of practical application. The present inventors have further verified that high level of safety is expectable

for this attenuated toxin and completed the present invention.

The objectives mentioned above can be attained by the present invention set forth below:

(1)   an adjuvant, comprising an attenuated toxin having a residual toxic activity of less than one-two thousandth (<1/2000) that of the natural toxin corresponding thereto, prepared by attenuating the natural toxin having serine residues, glutamic acid residues, and lysine residues in its amino acid sequence, or by attenuating the subunit thereof;

(2)   the adjuvant of (1), wherein the toxin is a mutant comprising the amino acid sequence of the corresponding natural toxin, wherein one or more amino acid residues are substituted, inserted, deleted, and/or added, and having an adjuvant activity;

(3)   the adjuvant of (1), wherein the toxin is a natural toxin;

(4)   the adjuvant of any of (1) to (3), wherein the toxin is a bacterial toxin;

(5)   the adjuvant of (4), wherein the toxin is selected from the group consisting of cholera toxin, pertussis toxin, heat-labile toxin of pathogenic $E.$ $coli$, Staphylococcus α toxin and β toxin, and thermostable hemolytic toxin of $Vibrio$ $parahaemolyticus$;

(6)   the adjuvant of (5), wherein the toxin is a natural cholera toxin having a toxic activity of less than one-two thousandth that of the corresponding natural toxin, prepared by attenuating a natural cholera toxin with formalin treatment;

(7)   the adjuvant of (6), wherein the toxic activity is less than one-ten thousandth that of the corresponding natural toxin;

(8)   a vaccine preparation comprising an adjuvant of any one of (1) to (7) and one or more vaccine antigens;

(9)   the vaccine preparation of (8), wherein the vaccine preparation is formulated for intranasal administration;

(10) the vaccine preparation of (8), wherein the vaccine preparation is formulated for oral administration;

(11) the vaccine preparation of (8), wherein the vaccine preparation is formulated for percutaneous administration;

(12) the vaccine preparation of (8), wherein the one or more vaccine antigens comprise antigens from one or more pathogenic

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microorganisms selected from the group consisting of influenza virus, rotavirus, measles virus, rubella virus, mumps virus, AIDS virus, *Bordetella pertussis*, diphtheria bacillus, *Helicobacter pylori*, enterohaemorrhagic *Escherichia coli* (EHEC), *Chlamydia*, *Mycoplasma*,

5   Malaria parasite, coccidium protozoa, and schistosome.

The present invention also relates to the use of an attenuated toxin as an adjuvant. The present invention further relates to the use of an attenuated toxin in the production of vaccine preparations.

The toxin composing the adjuvant of this invention is an

10 attenuated toxin that has substantially no toxic activity but still has the activity to enhance immunity. "Having substantially no toxic activity" is defined herein as having toxic activity that is less than one-two thousandth relative to that of the natural toxin. The activity of a representative bacterial toxin, such as cholera toxin,

15 *E. coli* heat-labile toxin, pertussis toxin, diphtheria toxin, or tetanus toxin, can be compared by, for example, using the ADP-ribosyltransferase activity as an index. That is, for example, when the ADP-ribosyltransferase activity is reduced to one-two thousandth, then it means that the toxic activity is reduced to one-two

20 thousandth.

Some technical papers so far reported described that ADP-ribosyltransferase was not detectable in various attenuated toxins. However, it may be improper to evaluate the toxic activity without any quantitative comparison, because the criterion is unclear in such

25 evaluation. Further, it is problematic because it does not allow comparison of results of two papers. In the present invention, a value of one-two thousandth of the natural toxic activity was adopted as an index in order to avoid possible confusion. Even when the toxin is of bacterial origin like cholera toxin or *E. coli* heat-labile toxin,

30 having a strong physiological activity, the high level of attenuation, wherein the toxicity is reduced to one-two thousandth that of the natural toxin, improve, to a great extent, the safety of adjuvants when injected to a human body, while retaining the adjuvant's immuno-stimulating activity.

35 In addition to the ADP-ribosyltransferase activity, the results obtained from bioassays utilizing physiological responses of animal

cells or experimental animals may also be used as an index of toxic activity, allowing the quantitative comparison.

In any case, it is preferable to verify that the toxicity is reduced to at least one-two thousandth that of the natural toxin by comparing the relative activities using an index that sensitively reflects the activity of toxin. Further, even if a sensitive index is used, it is also desirable in terms of safety to evaluate the toxic activity by the combined use of multiple indexes, each reflecting different mechanisms, because many toxins have pleiotropic physiological activities. Particular toxins and their activities are specified later. The toxic activity of the attenuated toxin of the invention is typically reduced to at least one-two thousandth that of the natural toxin, and is reduced to at least one-ten thousandth in preferred embodiments.

Attenuation of toxins can be attained by conventional methods. For example, chemical treatments and physical treatments can be used as techniques for the attenuation of toxins. The attenuation treatments can result in various structural changes, such as irreversible changes in three-dimensional structure, dissociation of subunits, or fragmentation of peptides. However, the adjuvant activity can be maintained in the toxin by retaining the above-mentioned three types of amino acid residues (e.g., serine, glutamic acid and lysine) in it. With such known methods, the condition where the toxic activity becomes at least one-two thousandth that of the natural toxic activity, as measured by any of the above-mentioned indexes representing the toxic activity, can be determined empirically. Specific conditions of attenuation are described later in detail.

The toxins of the present invention, specific examples of which are indicated later, must retain serine residues, glutamic acid residues and lysine residues contained in the amino acid sequence of the natural toxin. A preferred toxin of the present invention is a natural toxin. A high adjuvant activity is expectable; moreover, the steps required for screening mutants may be avoided by selecting a natural toxin.

In another embodiment, the toxin of the present invention can be a mutant which has the amino acid sequence of the natural toxin,

whereinone or more amino acid residues are substituted, deleted, inserted, and/or added, so long as the amino acid residues that are important for the adjuvant activity are retained. The mutant may include not only mutations in its amino acid sequence but also mutations
5   in sugar residues, which are other constituents of the toxin.

Functional groups of amino acid residues are important for protein function. For example, serine residue and threonine residue containing an OH group, glutamic acid residue and aspartic acid residue containing a COOH group, and lysine residue and arginine residue
10 containing an NH_2 group, often form the active center and play important roles in the expression of activity. Just as in the cases of many enzymes and functional proteins, it is believed that OH groups, COOH groups, NH_2 groups, and such in the amino acid residues forming the toxin molecule are closely associated with the expression of biological
15 activities including, for example, enzyme activity, activity of enhancing immunity, activity of binding to a receptor, etc. Furthermore, the number of amino acids may be another index showing the importance of OH group. For example, the total contents of the three types of amino acids, namely serine, threonine, and tyrosine
20 residue, each of which contains an OH group, in the polypeptides of cholera toxin, heat-labile toxin of human-type *E. coli*, and pertussis toxin are about 18%, 22% and 20%, respectively. The contents suggest the high possibility of their involvement in the active center. Accordingly, a recombinant mutant toxin that contains deletions or
25 substitutions with other amino acids of these amino acids closely related to the active center may easily cause remarkable changes in its three-dimensional structure. Thus, it is highly possible that it loses not only toxic activity but also the activity of enhancing immunity simultaneously. As an example supporting this proposition,
30 the present inventors have observed that in a recombinant mutant of *E. coli* heat-labile toxin, in which a lysine residue was substituted for the glutamic acid residue at amino acid position 112 from the N terminal end, while the toxic activity was reduced to about 1/2,860, the activity of enhancing the immunity was lost (K. Komase et al.,
35 Vaccine 16, 248-254, 1998).

Based on these findings, the present inventors selected three

amino acid residues, namely serine residue, glutamic acid residue, and lysine residue, from amino acid residues having the above-mentioned three types of functional groups as being important amino acid residues for adjuvant activity. The importance of these amino acid residues

5   for the activities of a variety of toxins has been reported previously.

Previous reports on the change of toxic activity associated with the substitutions of these amino acid residues are summarized in Table 1. Those reports described in Table 1 as having negative activity are reports in which substantially no residual toxic activity was

10   detected in the selected condition of measurement, but in which no quantitative comparison was carried out. Adjuvant activity was detedted in *E. coli* heat-labile mutant toxin but the activity of enhancing immunity was not sufficiently high. Further, 10 times as much as natural cholera toxin was required for a mutant cholera toxin

15   to give the same level of activity of enhancing immunity as natural cholera toxin. The indication "and others" means that other amino acid residues in addition to the residues indicated were also substituted. As shown, glutamic acid residue and serine residue are important residues that are often targets to be substituted with other

20   amino acid residues. However many reports describe that such substitutions frequently result in loss or declines of the adjuvant activity.

In the present invention, amino acid residues required for the maintenance of sufficient adjuvant activity includes, in addition

25   to these important amino acid residues that are targets for substitution, lysine residue containing an NH_2 group, which is a representative of basic amino acids. Lysine residues play an important role in the attenuation of toxin. For example, it is believed that in the formalin treatment, formalin attacks the ϵNH_2 group of lysine, and the formation

30   of a Schiff base at that site results in changes of three-dimensional structure, and thus the attenuation is achieved as a consequence. Accordingly, when lysine residues are retained, it is possible to readily attain a high degree of attenuation in which the toxicity is reduced to at least one-two thousandth that of the natural toxin.

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Table 1

type of toxin	substitution of amino acid residues (reference)	alteration of toxic activity and adjuvant activity	
E. coli	position112 Glu→Lys(1)	Y: 1/2860	—
heat-labile toxin	position 63 Ser→Lys(2)	A: negative	+
cholera toxin	position 63 Ser→Lys(2)	A: negative	±
	position 61 Ser→Phe(3)	C: 1/1000000	++
	position 112 Glu→Lys(4)	C: 1/1000000	++
pertussis toxin	position 129 Glu→Gly and others(5)	Y: 1/1000000	—
diphtheria toxin	position 162 Glu→Lys and others(6)	A: negative	—

Marker of toxic activity A: ADP-ribosyltransferase activity

C: spindle-forming test with CHO cells

Y: Y-1 cell morphologic transformation test

Study reports

(1) Komase et al., Vaccine 16, 248-254, 1998

(2) G. Douce et al., Infec. Immun. 65, 2821-2828, 1997

(3) S. Yamamoto Proc. Natl. Acad. Sci. USA, 94, 5267-5272, 1997

(4) S. Yamamoto J. Exp. Med. 185, 1203-1210, 1997

(5) M. Roberts et al., Infec. Immun. 63, 2100-2108, 1995

(6) T. Uchida et al., J. Biol. Chem. 248, 3838-3844, 1973

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However, a highly attenuated toxin, of which toxicity is reduced to at least one-two thousandth or, more preferably, at least one-ten thousandth that of the natural toxin and having a strong activity of enhancing immunity, is not predictable from the previous reports.

20　　　　For example, the cholera A subunit is thought to be responsible for the major toxic effect of cholera toxin. It has been expounded that the toxic activity is expressed when the strong ADP-ribosyltransferase activity of A subunit disturbs the balance of intracellular level of cAMP concentration in the cell permeated

25 by the toxin. The toxic activity of *E. coli* heat-labile toxin is also expressed by the same mechanism. A paper was published, wherein it

analyzed correlation between ADP-ribosyltransferase activity and the adjuvant activity of a recombinant of *E. coli* heat-labile toxin mutant. Based on the research result, it has been proposed that the two activities are closely related to each other and are not separable

5   from each other (N. T. Lycke et al., Eur. J. Immunol. 22, 2277-2281, 1992). Other reports also note that recombinant mutant toxins with reduced enzyme activity were accompanied with decreases in adjuvant activity (for example, J. D. Clements et al., Vaccine 6, 269-277, 1988, test sample; *E. coli* heat-labile toxin; W. J. Black et al.,

10   Science 240 (4852), 656-659, 1988, test sample; *Bordetella pertussis* toxin).

In other words, in previous theories, it has been believed that a high level of reduction of toxin activity does not lead to development of adjuvants with high safety, because the reduction is associated

15   with the decrease of the immuno-enhancing activity. And yet the attenuated toxin of the present invention demonstrates that the activity of enhancing immunity is substantially the same as that of the natural one, in some cases even when the toxic activity is reduced to at least one-two thousandth,, more preferably at least one-ten

20   thousandth, as compared with that of the natural one. These are novel findings first revealed based on the studies for long years by the present inventors.

It remains to be clarified why toxic activity and activity of enhancing the immunity are separable in the attenuated toxin. However,

25   a similar phenomenon has been found for a variety of vaccines using toxoid as the antigen. These attenuated toxins have been known to retain immunogenicity. An explanation can be offered based on the mechanism. For example, the following explanation can be given for the adjuvant activity of the attenuated cholera toxin. Since the

30   expression of toxic activity (for example, ADP-ribosyltransferase) requires a specific three-dimensional structure formed by a appropriately large-sized oligopeptide, even a minor change of three-dimensional structure results in loss of the activity. On the other hand, the activity of enhancing immunity can be expressed by

35   the whole toxin molecule or by a smaller peptide thereof, and the activity might be maintained with minor changes in the primary structure

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of the toxin protein.

It has been known that cholera toxin evokes various immuno-enhancing reactions when acting as an adjuvant. For example, (1) it enhances the permeability of antigen. It binds to a receptor
5 and activates helper T cells. It stimulates the proliferation of type-1 and type-2 helper T cells. Further, (2) it enhances the production of a variety of cytokines, for example, interferon-gamma, and interleukins 1, 4, 5, 12, etc. There is a possibility that in these immune responses, the toxin molecule effects without any structural
10 changes thereof in the case of (1), and the toxin molecule digested to peptide fragments show the effects in the case of (2). However, details of this remain to be resolved.

An antigen protein is processed to peptide fragments in series by the action of proteosomes. The resulting peptides are presented
15 by antigen-presenting cell. The presentation stimulates the subsequent series of immunoreactions: for example, the activation and proliferation of helper T cells, the production of a variety of cytokines, antibody production by B cells, and the like. There is a possibility that a similar mechanism to that described above works
20 for the expression of adjuvant effects of toxin.

The above-described possibility is a hypothesis at present. While the toxic activity is mainly due to a single mechanism, the adjuvant activity is exhibited by multiple mechanisms; therefore, it can be presumed that the attenuated toxin still retains the activity
25 of enhancing immunity even after losing the toxin activity.
Toxin:

Attenuated toxins which constitute the adjuvant of the present invention are derived from natural toxins produced by an organism such as bacterium, fungus, plant or animal. A bacterial toxin is a
30 particularly preferred toxin to be used as the adjuvant of the present invention. It is easy to produce bacterial toxin on a large scale and thus economically advantageous. Further, toxins derived from bacteria include, but are not limited to, toxins that have excellent adjuvant activity, e.g., cholera toxin. These include single proteins
35 and conjugated proteins. Specifically, in the context of the present invention, the term "toxin" refers to a proteinous substance having

toxic activity, which is produced by an organism in nature or a fragment
or a subunit thereof. However, in the context of the present invention,
the term "toxin" does not include low molecular toxins with a molecular
weight less than or equal to 500 with no expected adjuvant activity.
5   Further, toxin as defined in the present invention comprises neither
toxic substances (such as poisons), which are comprised in a part
of compounds synthesized by organic chemistry, nor toxic heavy metals.
　　　Illustrative examples of natural toxins contemplated by the
present invention include but are not limited to the following:
10 1. Bacterial toxins, including but not limited to, cholera toxin,
diphtheria toxin, pertussis toxin, staphylococcus α toxin,
staphylococcus β toxin, *Vibrio parahaemolyticus* thermostable
hemolytic toxin, heat-labile toxin of pathogenic *E. coli*, Shiga toxin,
pyocyanic enterotoxin A, etc.;
15 2. Fungus toxins, including but not limited to, canditoxin, fumigatoxin,
toxins of mushrooms, etc.;
3. Plant toxins, including but not limited to, ricin, etc.; and
4. Animal toxins, including but not limited to, snake venom, bee toxin,
toxins from Arthropoda (for example, scorpion venom), etc.
20 　　　Like pigments, antibiotics, and such, toxins are so-called
"secondary metabolites". Original physiological roles of toxins in
toxin-producing microorganisms, plants and animals often remain
unclear. Toxin production, as a characteristic nature of secondary
metabolite production, shows strain-specificity (i.e., is specific
25 to each individual in plant and animal) and not all organisms belonging
to a species are capable of producing the toxin. The frequency among
strains of the same species to produce the same toxin is not constant
and depends on the toxin. The same toxin can be produced by a sub-species
or a related species. Multi-component toxins, with toxins having
30 partially altered structure to a small degree, are often produced
by wild-type strains, related strains and artificially mutated
strains.
　　　Thus, a mixture of multiple toxins can be used as the adjuvant
of the present invention. Further, when a toxin molecule consists
35 of multiple subunits, one or more subunits that are required for the
adjuvant activity can be selected and used as the adjuvant of the

invention.   Toxins consisting of multiple subunits include, but are
not limited to, cholera toxin, pertussis toxin, diphtheria toxin,
heat-labile toxin of pathogenic *E. coli*, and canditoxin, etc.   The
subunit from a toxin, *per se,* generally exhibits adjuvant activity
5   (JP-A Hei 2-243633).   Accordingly, toxin of the invention can be such
a subunit.

Further, toxins to be utilized as the adjuvant of the invention
can be a mutant of the natural toxin.   The mutant may have a modified
structure in regard to the amino acid sequence, sugar chain and others
10   of the natural toxin.   Mutant toxins can be produced, for example,
by using artificial mutant strains generated from toxin-producing
wild-type or related strains by treating them with mutagenizing agents.
Such a toxin is herein referred to as "artificial mutant toxin".   The
mutant toxin can be also produced by recombinant cells, to which mutant
15   toxin-producing   capacity   has   been   given   by   utilizing   gene
recombination   techniques.   The   latter   is   herein   referred   to   as
"recombinant mutant toxin".

Because a mutant toxin has a partially altered structure as
compared with that of the natural molecule, it generally loses some
20   of the original properties of the toxin as a result of such structural
changes.   However, there are cases in which the activity of enhancing
immunity is retained.   When the toxic activity of mutant toxin is
sufficiently low, e.g., reduced by a factor of at least one-two
thousandth relative to that of the natural one, it can be used as
25   an attenuated toxin for the adjuvant of the present invention provided
its activity of enhancing immunity has been verified.   Otherwise, the
mutant toxin is further attenuated by using any of the above-mentioned
chemical and physical treatments to give rise to a usable adjuvant.
It is preferable that the mutant toxin is stable under the conditions
30   where the toxin-containing vaccine is to be used.   Further, mutants
wherein the restoration of toxic activity is limited or negligible
are preferable.   "Restoration of toxin activity" refers to a phenomenon
in which the toxic activity, which has been reduced greatly by a variety
of treatments, is recovered as time progresses after the treatment.
35   There is no particular limitation on regions where the structures
are to be changed in an artificial mutant toxin, so long as the specified

three types of amino acid residues remain unchanged and the mutant still has the adjuvant activity. For example, such structural alteration is exemplified by one or more alterations of amino acid residue of oligopeptide moiety, sugar residue of oligosaccharide
5   moiety, organic acid moiety, and such in the toxin molecule.

It can be difficult to artificially choose an amino acid residue or sugar residue to be mutated in the artificial mutant. Therefore, in general, it is difficult to select the type of mutation *a priori*. However, the reduction of toxic activity and the activity of enhancing
10   immunity are easily verified during research and therefore mutants that meet the requirements of the present invention can be obtained. Further, it has the advantage that mutants with alterations of unexpected amino acid residues can be isolated.

On the other hand, it is possible to choose amino acid residues
15   or sugar residues to be mutated and to intentionally introduce a desired mutation in regard to the recombinant mutant toxin. Specifically, such alterations include one or more alterations of amino acid residue of oligopeptide moiety, sugar residue of oligosaccharide moiety, organic acid moiety, and such in the toxin molecule. The adjuvant
20   of the invention also includes oligopeptide fragments such as peptide fragments playing an important role in the expression of the activity of enhancing immunity.

<u>Production of natural toxin and mutant toxin:</u>
25   Natural toxins can be obtained as the products of wild-type strains. Alternatively, the toxins can be obtained as the products of a variety of artificial mutant strains such as overproducing strains. Or the toxins can be obtained as the products of recombinant cells, in which the genes of the wild-type strain and such from the same
30   species or a different species, have been introduced by genetic engineering. Further, the production of the toxins can be conducted by procedures of chemical synthesis.

Bacterial toxins utilized in the present invention can be extracted, separated, purified and produced, for example, using a
35   culture of toxin-producing bacterium as a starting material and combining conventional methods. In regard to cholera toxin, an

exemplary method is as follows. Toxin-producing cholera bacterium (*Vibrio chorela*) is cultured at 36°C for 18 hours. The culture liquid is used as a culture seed for large-scale cultivation on agar plates or liquid culture media. After the culture is complete, the culture

5 supernatant (in the case of liquid culture) is concentrated by ammonium sulfate precipitation or ultrafiltration. The toxin is then purified using one or more conventional methods such as column-chromatography using Sephadex and such, ultracentrifugation, gel electrophoresis, etc. The toxic activity in a sample is assayed during cultivation

10 or purification by the procedure described later.

In regard to pertussis toxin, an exemplary production method is as follows. A pertussis bacterium (*Bordetella pertussis*; Tohama; phase I) is cultured in Bordet-Gengou medium to give a culture seed. The seed is transferred into Cohen-Wheeler medium and cultured for

15 48 hours and the culture supernatant is used as a starting material. The culture supernatant is loaded onto a column of hydroxyapatite. The column is washed with 0.1 M phosphate buffer (pH 7.0), and subsequent elution is performed with 0.1 M phosphate buffer (pH 7.0) containing 0.5 M NaCl. The toxin is further purified by column chromatography,

20 using CM-Sepharose or other carriers on which adequate affinity ligands are immobilized. The toxic activity of a sample is assayed during cultivation or purification by conventional methods.

In addition, toxins produced by mushrooms can be extracted and purified from mushrooms that grow naturally or that are cultivated.

25 A illustrative method for producing animal toxin is as follows: a toxin-producing animal, for example a snake, is bred for the toxin. The toxin is collected from the toxin-producing organ in the mouth. As the matter of course, if commercially available, the toxin may be purchased from the suppliers. Production processes for the toxin

30 and quality thereof can be variously altered as far as the alterations meet the standard for the corresponding biological preparations and are in accordance with related laws and regulations.

An artificial mutant toxin can be produced by an artificial mutant strain derived from toxin-producing wild-stain. The artificial

35 mutant strain is generated from the wild-strain by the treatment with a mutagenizing agent, for example. Known methods of mutagenesis

include, but are not limited to, chemical treatment, using chemical agents such as N-methyl-N'-nitro-N-nitrosoguanidine, nitrosourea, nitrogen mastard, etc., and physicochemical treatments, such as ultraviolet light irradiation, radiation exposure with cobalt-60 or

5 the like, heating, etc. Culture of artificial mutant strains, purification of toxins from the mutant strains, and verification of the activity thereof are carried out essentially in the same manner as for the wild-type strains.

A recombinant mutant toxin can be produced by recombinant cells.
10 Methods for the production of such cells are conventional in the art. The following is an illustrative example of a method for producing a recombinant heat-labile toxin mutant of *E. coli*. A toxin-overproducing recombinant strain is prepared from a strain producing heat-labile toxin separated from patients (*Escherichia coli*
15 1032 strain), for example, by using the method described by Tamura et al. (S. Tamura et al., Vaccine 12, 1083-1089, 1994). As the first step, separation treatment is carried out to obtain a plasmid (65 kbp) containing both genes encoding heat-labile toxin and thermostable toxin. The plasmid is treated with restriction enzymes and such to
20 cut out the heat-labile toxin gene (6.7 kbp). The resulting fragment is ligated into a plasmid, such as pBR322 or the like. The fragment is amplified by PCR or the like. The heat-labile toxin genes are then ligated into other expression vectors. Subsequently, site-specific mutagenesis is performed on the vector, by the method described by
25 Ho et al. (S. N. Ho et al., Gene 77, 51-59, 1989) for example. The fragment of interest is then amplified by PCR method, using a pair of selection primers in order to give a recombinant *E. coli* strain producing recombinant mutant toxin. In the next step, the bacterial strain is cultured according to the method described by Clements et
30 al. (J.D. Clements et al., Infect. Immun. 24, 760-769, 1979), for example. The protein of interest is purified from the culture supernatant by ultrafiltration, ammonium sulfate precipitation, agarose gel chromatography (elution with a buffer containing galactose) and others. It is thus possible to obtain recombinant mutant
35 toxins derived from the heat-labile toxin of *E. coli* that is infectious to human. Alternatively, natural toxins can be obtained when the

site-specific mutagenesis is not conducted in the process described above.

It is possible to replace amino acid residues with other residues at desired positions in the amino acid sequence of a toxin, for example,

5　by using site-specific mutagenesis as described above. On the other hand, specific amino acid residues, for example a glutamic acid residue, may remain unchanged at particular positions. Sugar residues can also be treated basically in the same manner. The mutants obtained are screened to select those having adjuvant activity, which selection

10　yields the mutant toxins of the invention. Each mutant may exhibit the toxic activity at any level. In other words, when the adjuvant activity is satisfactorily maintained but the level of toxic activity of the mutant is comparable to that of the natural one, then the mutant toxin can be used as the adjuvant after proper attenuation treatment

15　as used for the natural one. Otherwise, if the adjuvant activity is satisfactorily maintained and the level of toxic activity of the mutant is preferably low, then the mutant toxin can be used as the adjuvant of attenuated toxin without any modification or after subjecting to some attenuation treatment. Although trial-and-error experimental

20　approaches are required to obtain the mutants, such approaches generally have the advantage that the resulting recombinant mutant toxins hardly revert to the original toxic activity.

Method for attenuating toxin:

25　Before using the adjuvants of the invention, one must first verify whether or not the toxins produced by the wild-strain, artificial mutant strain, or recombinant mutant strain have the activity of enhancing immunity after being attenuated. The toxins can be attenuated by any conventional method. Examples of suitable

30　attenuation methods are described below. The term "attenuation" means herein that the toxic activity is reduced by using any means and it should not be construed as being limited to the examples. However, these methods are convenient and reliable to provide the effect of treatment.

35　All of these attenuation methods are publicly known methods used for converting to toxoids. In the present invention, however, it is

required to attain high levels of attenuation in which toxic activity is reduced to one-two thousandth. Accordingly, more extreme conditions are required for the attenuation in the present invention, although the methods to be used here are based on the same principles

5   as the publicly known methods. Specifically, for example when cholera toxin is treated with formalin, temperature for the treatment is 5 to 30°C (ordinary 5°C), the concentration of formalin is 0.5 to 0.8% (ordinary 0.3%), and the duration of treatment is 20 to 70 days (ordinary about 7-14 days), or the like.

10

[1] Chemical method

      The toxin is dissolved at an adequate concentration in a buffer. While the pH is adjusted to be within a range where the toxin is stable

15   (typically, pH 5 to 8), an agent, for example, formalin, glutaraldehyde, phenol, iodine, acid anhydride, or a detergent such as bile acid with the adequate concentration (typically, 0.01 to 1.0%) is added stepwise. Then the solution is incubated at an adequate temperature (for example, 5°C). After incubation, the agent is removed by any suitable method

20   such as dialysis. The residual toxic activity is then assayed. If desired, the treated toxin is incubated again at a temperature at which the attenuated toxin will be used practically, for example, at 37°C, in order to secure that the toxic activity does not recover. The attenuated toxin of interest is thus yielded. This method is based

25   upon the previous method widely used for the toxoid conversion, and is also suitable for large-scale production. However, it is preferable to use a method with which the toxic activity does not recover. There are some conventional methods used for this purpose. Exemplary methods include but are not limited to (1) the simultaneous addition of lysine in a quantity corresponding to the concentration of the agent for

30   the attenuation treatment, for example, formalin, and (2) the performance of a reduction treatment ¥ after the attenuation.

[2] Physical method

      The toxin is dissolved at an adequate concentration in a buffer.

35   While the pH is adjusted to a pH at which toxic activity is generally lost, including an acidic pH (for example, pH 2 to 4) or alkaline

pH (for example, pH 8 to 11), the solution is incubated at an adequate temperature.   Alternatively, the solution is incubated at a temperature at which the toxic activity is generally lost (for example, at a temperature of 40°C or higher).   Otherwise, the toxin is sonicated

5.   at an adequate wavelength, or irradiated with electromagnetic wave. The residual toxic activity of the sample is assayed before, during and after the treatment.   If desired, the attenuated toxin is re-incubated at an adequate temperature, for example, at 37°C, to secure that the toxic activity does not recover.   If the activity of

10   enhancing immunity is confirmed to be sufficiently high, then the preparation of attenuated toxin is completed.   The chemical and physical treatments can be properly combined for attenuation.

Method for verifying residual toxin activity

15   Residual toxic activity can be assayed by any conventional method (see, for example, "Handbook for vaccination," Ed. Research Association, National Institute of Health of Japan, Maruzen, 1994; "Protein toxin," 1st and 2nd volumes, Ed., R. Tamura et al., Kodansha Scientific, 1972, etc.).   The methods can be categorized as follows:

20   enzyme activity assays, physiological-response assays using animal cells, physiological-response assays using animals, and evaluation methods utilizing survival and death of experimental animals, etc. The specific assay method to be used depends on the type of toxin. The following examples describe representative indexes for selecting

25   such a method.   Specific procedures for these assays are known.   The comparison of toxic activity is not limited to these indexes, but as the matter of course the comparison of toxin activities before and after the attenuation should be carried out using the same index.

30   (Bacterial toxin)
Cholera toxin, heat-labile toxin of pathogenic *E. coli*
        ADP-ribosyltransferase activity
        Accumulation of cAMP
        Y-1 cell morphologic transformation

35   Weight loss in mouse
        Elongation of CHO cell

Pertussis toxin

      ADP-ribosyltransferase activity

      Elongation of CHO cell

      Leukocyte increasing activity

5   Histamine sensitizing activity

      Weight loss in mouse

      Elongation of CHO cell

Diphtheria toxin

      ADP-ribosyltransferase activity

10   Survival of guinea pig

      Rabbit cutaneous sensitization activity

Tetanus toxin

      ADP-ribosyltransferase activity

      Death of guinea pig

15   Rabbit cutaneous sensitization activity

Staphylococcus α toxin, staphylococcus β toxin

      Hemolytic activity

Shiga toxin

      Hemolytic activity

20 Pseudomonas enterotoxin A

      ADP-ribosyltransferase activity

Vibrio parahaemolyticus thermostable hemolytic toxin

      ADP-ribosyltransferase activity

      Hemolytic activity

25 (Fungus toxin)

Canditoxin, fumigatoxin,

      Mouse killing activity

(Animal toxin)

Snake venom

30   Cholinesterase activity

      Cholinesterase-inhibiting activity

      Phospholipase A2 activity

      Blood coagulation activity

      Platelet aggregation activity

35   Anticomplement activity

Bee toxin

N-acetyl glucosaminidase activity

Hyaluronidase activity

In assaying toxin activity, even when the residual toxic activity is not detected by one method, a different result can be obtained by an alternate method. For example, there was a case where the toxic activity was detected by Y-1 cell morphologic transformation test although substantially no residual activity of attenuated cholera toxin was recognized by ADP-ribosyltransferase activity (for example, K. Komase et al., Vaccine 16, 248-254, 1998). In another case, although ADP-ribosyltransferase activity was substantially not detectable in a recombinant mutant of *E. coli* heat-labile toxin in which the amino acid residue was replaced with lysine residue at amino acid position 7 from the N terminus, the attenuated toxin induced diarrhea. In other words, the toxic activity of *E. coli* heat-labile toxin can be represented by ADP-ribosyltransferase activity, but there is a second minor toxic activity that is expressed by a different mechanism. It is thought that when the first activity is markedly reduced in the recombinant mutant, then the second toxic activity increases relatively and as a consequence the second one becomes detectable. In general, assay methods with living cells or living bodies tend to show higher sensitivity in detecting toxic activity. The above-mentioned mechanism is considered to be involved in this detection.

Accordingly, in general, the comparison of toxic activity can be performed more reliably by using a sensitive method based on the response of living cells or living bodies. It is also preferable to evaluate the residual toxic activity by multiple assaying methods. In Examples described herein, the residual toxic activity of cholera toxin was evaluated by the combined use of an assay for receptor-binding activity, a Y-1 cell morphologic transformation test, a mouse foot swelling test, and a method involving the intraperitoneal administration to a mouse. Enzyme immunoassay (ELISA) using anti-toxin antibody is also a useful index representing the toxic activity. With any method, the most important thing is to reduce the toxic activity to at least one-two thousandth that of the natural toxin, in principle, based on an index that directly reflects the toxic activity.

The adjuvant of the invention comprises the above-mentioned attenuated toxin alone or in combination with a preservative, stabilizer, and/or a conventional adjuvant. In general, the safety of an adjuvant is judged by repeatedly measuring safety. In addition,
5   it is preferable to evaluate the safety by adopting a method using a living cell or living body as one of the indexes. As a matter of course, a conventional safety test, in which acute toxicity is examined by administering the adjuvant into experimental animals, should be conducted. Further, the absence of irritability on the skin and mucous
10 membrane is an important safety index for oral administration, percutaneous administration and intranasal administration.

Adjuvant activity:

The present invention utilizes, as the adjuvant, an attenuated
15 toxin in which the toxic activity is attenuated to at least one-two thousandth that of the natural toxin, as determined by a particular attenuation method, and in which the adjuvant activity is maintained. The adjuvant effect can be maintained at a high degree in the toxin by retaining the three types of amino acid residues that are specified
20 in the summary above, namely serine, glutamic acid, and lysine. However, it is necessary to test whether or not the adjuvant activity is reduced during the process of treatment to reduce the toxic activity. The adjuvant activity can be verified by immunizing an animal with a substance to be compared for the activity, together with an
25 immunological antigen, and then by observing the increase in titer of antibody against the immunological antigen. It is possible to compare the adjuvant activity by using a uniform condition like using animals of the same line and using identical immunological antigen.

30 Usage pattern:

There is no particular limitation on the usage pattern for the attenuated toxin of the present invention as an active ingredient in a vaccine. In other words, the adjuvant can be used with various known appropriate usage patterns. For example, the adjuvant may be
35 a physically mixed preparation or a complex chemically linked with an antigen protein. In addition, the adjuvant can be incorporated

together with a vaccine in a carrier such as liposome.

The attenuated toxin of the invention can be used concurrently together with one or more conventional adjuvants. Example 5 shows the combined use of the attenuated cholera toxin adjuvant of the present invention with *E. coli* heat-labile toxin B subunit that is a conventionally known adjuvant. When used together with *E. coli* heat-labile toxin B subunit, the attenuated toxin adjuvant elevated the immunological activity in mucous membrane and further exhibited a synergistic effect. Those skilled in the art can readily find a preferable combination of adjuvants using routine experimentation, using publicly known methods. Based on the result obtained, it is possible to reduce adverse side reactions and enhance the immunoreactivity, for example, by reducing the amount of antigen or the other adjuvant.

Vaccine:

New vaccine preparations are provided by utilizing the adjuvants of the present invention. A vaccine preparation in the context of the present invention comprises the inventive adjuvant and any immunogenic antigen, and encompasses "vaccines" in both narrow and broad senses. Specifically, the vaccines of the present invention include vaccines in the narrow sense, such as those that are effective against infection by a virus, bacterium, fungus, protozoan, or other microorganisms capable of infecting to human or other animals. Illustrative examples of such vaccines include, but are not limited to, influenza vaccine, pertussis vaccine, purified pertussis-diphtheria-tetanus toxoids combined vaccine, Japanese encephalitis vaccine, hepatitis A vaccine, hepatitis B vaccine, rotavirus vaccine, measles vaccine, rubella vaccine, mumps vaccine, measles-rubella-mumps trivalent vaccine, measles-rubella divalent vaccine, vaccine against haemophilus influenzae, etc. The vaccines also include tuberculosis vaccine, vaccine against multi-drug resistant Staphylococcus aureus (MRSA), *Helicobacter pylori* vaccine, enterohaemorrhagic *Escherichia coli* (EHEC) vaccine, salmonella vaccine, *Chlamydia* vaccine, *Mycoplasma* vaccine, AIDS vaccine, malaria vaccine, coccidium vaccine, and schistosome vaccine. Vaccines in the

broad sense include those vaccines that are effective in the prevention and treatment of non-infectious diseases such as anti-cancer vaccine, vaccine of contraception, anti-gastric ulcer vaccine, anti-diabetic vaccine and anti-arteriosclerotic vaccine. Vaccines can be

5　administered by injection, orally, percutaneously, intranasally or by other methods.

The vaccines of the present invention include various vaccines that can be categorized based on the types of methods to produce them. Specifically, the vaccines of the present invention include attenuated

10　live vaccines, inactivated vaccines, component vaccines, vaccines using DNA, etc. The "vaccines using DNA" include both vaccines containing a DNA fragment integrated in a carrier, such as plasmid, and vaccines used in combination with ribozymes or antisense oligonucleotides, and the like. These vaccines can be used for

15　prevention or treatment. The vaccines also include recombinant vaccines containing an antigen, which is the active ingredient of vaccine, produced in living cells engineered by gene recombination techniques. These vaccines may be monovalent vaccines or combined vaccines. Illustrative production methods and usage patterns of the

20　vaccines are as follows.

- Influenza vaccine: a split vaccine which contains hemagglutinin (HA), neuraminidase (NA), nuclear protein (NP), matrix protein (M), or a part thereof, obtained by (a) growing viruses in embryonated

25　eggs or in Vero cells or by other animal cell culture techniques and (b) decomposing the virus particles into respective components thereof with ether, detergent or the like and purifying the virus components, or by genetic engineering or chemical synthesis and which is administered by injection, orally, percutaneously or

30　intranasally; alternatively, a DNA vaccine containing DNA fragments of genes encoding these proteins to be given by intranasal route.

- Pertussis vaccine: an inactivated vaccine that is obtained by culturing *Bordetella pertussis*, treating the culture supernatant or bacteria by salting-out, ultracentrifugation, and the like to

35　extract constituents of interest, and detoxicating with formalin; alternatively, a recombinant vaccine containing *Bordetella*

pertussis toxin (PT), hemagglutinin (FHA), 69K membrane protein, or a partial peptide thereof, each of which is prepared by genetic engineering or chemical synthesis.

- Pertussis-diphtheria-tetanus combined vaccine: a trivalent vaccine prepared by mixing pertussis vaccine with diphtheria toxoid and tetanus toxoid, which is administered by injection, orally, percutaneously or intranasally.

- Japanese encephalitis vaccine: a vaccine containing antigen proteins that are obtained by (a) growing viruses in the mouse brain or in Vero cells using animal cell culture techniques, (b) purifying the virus particles by ultracentrifugation or with ethyl alcohol, and (c) inactivating with formalin; alternatively, antigen proteins can be obtained by genetic engineering or chemical synthesis.

- Hepatitis B vaccine: a plasma vaccine that is obtained using blood collected from hepatitis B carriers as raw material, and separating and purifying HBs antigen by salting-out, ultracentrifugation, and others; alternatively, a recombinant vaccine containing antigen portions obtained by gene engineering or chemical synthesis.

- Measles vaccine: a live vaccine of attenuated virus that is prepared by growing the viruses in culture cells such as chicken embryonic cells or in embryonated eggs; alternatively, a vaccine containing a part of the virus or a recombinant vaccine containing the protective antigen prepared by gene engineering or chemical synthesis.

- Rubella vaccine: a vaccine containing viruses grown in culture cells, such as chicken embryonic cells or in embryonated eggs, part of the virus or the protective antigen prepared by genetic engineering or chemical synthesis.

- Mumps vaccine: an attenuated live vaccine containing viruses grown in culture cells, such as rabbit cells or in embryonated eggs, part of the virus, or the protective antigen prepared by gene engineering or chemical synthesis.

- Measles-rubella-mumps vaccine; a trivalent vaccine that is obtained by combining measles vaccine, rubella vaccine, and mumps vaccine.

- Measles-rubella vaccine; a divalent vaccine that is obtained by combining measles vaccine and rubella vaccine.

- Rotavirus vaccine: a vaccine containing viruses grown in culture

cells such as MA104 cell, viruses collected from patient's feces, part of the virus, or protective antigen prepared by genetic engineering or chemical synthesis.

- *Mycoplasma* vaccine: a vaccine containing *Mycoplasma* grown in medium
5   for *Mycoplasma*, a part thereof, or protective antigen prepared by genetic engineering or chemical synthesis.

- AIDS vaccine: a vaccine containing viruses grown in culture cells or viruses obtained from patients, part thereof, or protective antigen prepared by genetic engineering or chemical synthesis;
10   alternatively, a DNA vaccine containing effective DNA fragments.

- *H. pylori* vaccine: a vaccine containing, as antigens, lysate of cultured *H. pylori*, or urease, heat shock protein, toxin and others separated from *H. pylori* culture; alternatively, a vaccine comprising these antigen proteins produced by genetic engineering,
15   and administered by injection, oral inoculation, percutaneous inoculation or intranasal inoculation.

Properties of vaccine:

     The vaccines above may be provided as liquid forms or powdered
20 forms.

     When each of these vaccines is administered together with an attenuated toxin adjuvant of the present invention, the liquid form of the vaccine is often more suitable for intranasal inoculation (intranasal spray, intranasal instillation, spread, etc.) and
25 injection. Further, the intranasal inoculation can be performed with powder spray. The vaccine preparations of the invention can also be formulated with a conventional stabilizer or preservative. An illustrative stabilizer includes, but is not limited to, gelatin and dextran of about 0.2%, sodium glutamate of 0.1 to 1.0%, lactose of
30 about 5%, and sorbitol about of 2%. Preservatives include about 0.01% thimerosal, phenoxyethanol of about 0.5%, and about 0.1% β-propiolactone.

Mixing ratio of attenuated toxin:

35   The mixing ratio between a vaccine antigen and an attenuated toxin adjuvant of the present invention can be, for example, 1:0.0001

to 1:10000 (by weight ratio) in vaccine preparations in accordance with the present invention. However, this range is merely illustrative and is not limiting on the vaccines of the present invention. A suitable ratio can be routinely selected depending on the type of vaccine.

5   Methods required for selection are known to those skilled in the art. As seen in the Examples below, an inventive adjuvant comprising an attenuated toxin derived from a natural one in accordance with the present invention exhibits activity of enhancing immunity, the level of which is the same as that of the natural one when used at the same

10 quantity. On the other hand, with a conventional recombinant mutant, it is often necessary to inoculate with a larger quantity to attain the same level of immuno-enhancing activity as that achieved with the natural toxin.

15 Mixing procedure of attenuated toxin:

The vaccine preparation of the present invention can be prepared by combining the above-mentioned vaccine with the attenuated toxin adjuvant of the invention at an adequate mixing ratio. The preparation must be done in a strictly sterile condition. Each of the raw materials

20 must also be prepared under a completely sterile condition. It is desirable to remove contaminated proteins, i.e., those that have no vaccine activity and that act as pyrogens or allergens, as much as possible. Methods to achieve the treatment are conventional and known to those skilled in the art. The vaccine preparation of the invention

25 can be effective even when the vaccine antigen and the attenuated toxin of the invention are separately prepared as pharmaceutical preparations and then the two are combined with each other at the time of inoculation or the two are inoculated at about the same time.

30 Vaccination method:

Any conventional method can be utilized for dosage pattern of the vaccine preparation in accordance with the present invention.

The dose is preferably about 5 to about 50 μl for intranasal inoculation to mouse; preferably about 0.1 to about 1.0 ml for the

35 inoculation to human by intranasal administration or injection. Percutaneous inoculation can be performed by fixing the vaccine

composition to a carrier and then contacting with the skin. The dose
of antigen protein in a vaccine composition generally ranges from
about 0.1 ng to about 100 mg per administration, preferably from about
1 µg to about 1 mg per administration. On the other hand, the amount

5   of adjuvant to be formulated is determined depending on the type and
dose of immunological antigen to be used. The carrier for the vaccine
composition can be an absorptive carrier, such as lint, and a
conventional carrier for applying the dose as a paste or spray.

Oral, percutaneous or intranasal inoculation is highly preferred

10   for the vaccines exemplified below, both in terms of effectiveness
of vaccine and convenience of inoculation procedure. Examples of such
vaccines include, but are not limited to, influenza vaccine, pertussis
vaccine, diphtheria vaccine, rotavirus vaccine, measles vaccine,
rubella vaccine, mumps vaccine, *Helicobacter pylori* vaccine,

15   enterohaemorrhagic *Escherichia coli* (EHEC) vaccine, *Chlamydia* vaccine,
mycoplasma vaccine, coccidium vaccine, AIDS vaccine, malaria vaccine,
and schistosome vaccine, etc.

These vaccines can be administered alone or in combination with
other agents, by mixing more than two of them and inoculating them

20   simultaneously, for example, like pertussis-diphtheria-tetanus
trivalent vaccine or measles-rubella divalent vaccine. One of the
reasons why oral and intranasal inoculations are preferred is that
mucous membranes of the respiratory tract and digestive tract are
important sites of early stage infection. Such inoculations can

25   prevent infection at a stage as early as possible by activating the
immunogenic system in the local mucous membrane with the inoculation
of a vaccine comprising an appropriate adjuvant possessing potent
immuno-stimulating activity. Further, for some vaccinations, such
as vaccinations against Malaria *Plasmodium*, which are typically

30   performed in regions without sufficient medical facilities, it is
advantageous to select vaccination routes such as oral, percutaneous
and intranasal inoculation routes because such vaccines can be
administered without the help of medical staff such as physician or
nurse. In addition, percutaneous inoculation is desirable, because

35   the vaccination can be halted as may be demanded when adverse side
effect or the like encounters.

Brief Description of the Drawings

Figure 1 is a graph showing the binding capacity of attenuated cholera toxin to ganglioside GM1.

5   This figure shows an example of a confirmation test for attenuation, which was conducted during the process of producing an adjuvant of the invention by attenuating cholera toxin with formalin treatment. In this figure, the ordinate axis indicates the binding capacity to ganglioside GM1, which is the receptor (O.D. at 410 nm

10   in ELISA), and the abscissa axis indicates toxin concentration (ng/ml).

Figure 2 is a graph showing the effect of attenuated cholera toxin to enhance production of anti-influenza virus antibody in the nasal mucous membrane by the secondary response.

This figure shows the antibody titer in nasal wash, resulting

15   from the intranasal administration of a vaccine preparations of the present invention comprising an influenza vaccine antigen. The ordinate axis indicates attenuated cholera toxins treated under a variety of conditions and the abscissa axis indicates the concentration (μg/ml) of anti-HA IgA antibody in the nasal washes.

20   Figure 3 is a graph showing the effect of attenuated cholera toxin to enhance the production of anti-influenza virus antibody in the serum by the secondary response.

This figure shows the antibody titer in the serum, resulting from the intranasal administration of a vaccine preparation of the

25   present invention comprising an influenza vaccine antigen. The ordinate axis indicates attenuated cholera toxins treated under a variety of conditions and the abscissa axis indicates the concentration (μg/ml) of anti-HA IgA antibody in the serum.

Figure 4 is a graph showing the immuno-stimulating activity of

30   attenuated toxin.

This figure shows the antibody titer in the nasal wash or in the serum in the secondary response, resulting from the intranasal administration of an influenza vaccine of the present invention, containing cholera toxins of which residual toxin activities are

35   different to each other as the adjuvants. The ordinate axis indicates a relative value (■) of anti-HA IgA antibody titer in the nasal wash

of the experiment group, where attenuated cholera toxin was used, to anti-HA IgA antibody titer in control group, where natural cholera toxin was used, or a relative value (○) of anti-HA IgG antibody titer in the serum of the experiment group, where attenuated cholera toxin

5   was used, to anti-HA IgG antibody titer in control group, where natural cholera toxin was used.   The abscissa axis indicates a relative value of residual toxic activity of attenuated cholera toxin used in the test to the activity of natural toxin (measured by Y-1 cell morphologic transformation test).

10   Figure 5 is a graph showing the synergistic effect of dual adjuvants.

        This figure shows immunological responses resulting from the intranasal administration of an influenza vaccine that contains the attenuated cholera toxin of the invention and a conventional *E. coli*

15   heat-labile toxin B subunit as the adjuvants.   The ordinate axis indicates the type and amount of adjuvant used in the test and the abscissa axis indicates the degree of immunological response measured by mouse foot swelling test.

20   Best Mode for Carrying out the Invention

        Examples of the present invention are illustrated below, but the present invention is not to be construed as being limited thereto.

Example 1 - Preparation of cholera toxin:

25   The cholera toxin was produced according to the method described by Finkelstein et al. (R. A. Finkelstein et al. J. Infect. Dis., 121, Suppl., S63, 1970).

        A cholera toxin-producing bacterium (*Vibrio chorela*; Inaba type 569B strain) was cultured in a semi-synthetic casamino acid medium

30   (liquid medium containing glucose) designed by Finkelstein et al., at 30°C for 20 hours. After the culture was completed, the culture supernatant was subjected to ultrafiltration, using a filter having pores that the cholera toxin molecule (molecular weight; 86,000 Da) could freely go through. A small amount of aluminum hydroxide gel

35   was added to the filtrate. The gel was allowed to adsorb the toxin and then was collected by centrifugation. The toxin was eluted from

the gel with 10% phosphate-10% citrate (pH 7.6), while incubating
at 30 to 35°C. The eluted liquid was dialyzed against an aqueous
solution containing 0.1 M citrate. Then, the solution was loaded onto
a column of DEAE-Sephadex and the toxin was eluted with phosphate

5　buffer containing 0.1 to 0.2 M saline (hereafter abbreviated as PBS).
Subsequently, the sample was loaded onto a column of Sephadex G-75
and the toxin was eluted with PBS. The resulting sample was subjected
to gel electrophoresis. Only the presence of the cholera toxin as
a single band was detected. The purity was about 95%. The yield was

10　250 mg/100L culture liquid.

Example 2 - Attenuation of cholera toxin:

　　　The purified cholera toxin obtained in Example 1 was dissolved
in 0.01 M PBS (pH 7-8). 0.05 M lysine was added to the solution. Then

15　formalin was added dropwise to each aliquot of the solution at a final
concentration of 0.1, 0.3, 0.5, 0.6, 0.8, or 1.0%. The resulting
solutions were incubated at 30 to 40°C for 7 to 96 days. Samples were
taken during the incubation. Immediately, the collected samples were
dialyzed against 20 times as much volume of PBS to remove formalin.

20　The dialyzed solution was then sterilized by filtration to give
attenuated cholera toxin. The time course of the activity change was
investigated by using part of the samples collected during the treatment.
Figure 1 shows an example where the sample collected on the twelfth
day of the formalin treatment was assayed by the method for the residual

25　toxic activity, utilizing as an index the binding ability to ganglioside
GM1, which is a receptor for cholera toxin B subunit (a subunit having
the binding activity to target cells of toxin; A subunit is responsible
for the toxic activity). The result shows that the binding ability
of the attenuated toxin to ganglioside GM1 is reduced to about 1/15

30　and 1/100 of the natural one, when the toxin was treated with 0.3%
and 0.5% formalin for 12 days, respectively.

　　　The Y-1 cell morphologic transformation test was utilized to
assay the residual toxic activity of a variety of samples that were
subjected to attenuation treatment under various conditions. Part

35　of the result is indicated in Table 2. The result shows that the residual
activity is reduced by a factor of about 1/1780 to about 1/114000

when the toxin was treated with 0.3% and 0.5% formalin for 12 to 96
days.　Acute toxicity test was conducted in mice by using the attenuated
cholera toxin prepared above.　The attenuated cholera toxin, indicated
in Table 2, in which the residual toxic activity was 1/7000 of the
original activity or lower, did not show toxicity when administered
intraperitoneally at a dose of 30 mg/kg.　Accordingly, the toxic
activity can be reduced to one-two thousandth or lower (using acute
toxicity in mouse as an index) by treating the toxin with 0.3% formalin
at 35°C for 12 days or more.

Table 2

Residual toxic activity of formalin-treated cholera toxin
(according to the result obtained by Y-1 cell morphologic
transformation test)

Formalin Treatment		Residual Toxicity (ED50)		Attenuation Rate
Concentration (%)	Days	Dilution (4^n)	(4^n)	Approximate Value of Attenuation Magnification
0.3	12	2.0 ± 0.6	6.7	10,800
	24	2.3 ± 0.6	6.4	7,130
	48	2.7 ± 0.6	6.0	4,100
	72	3.3 ± 0.6	5.4	1,780
	96	3.3 ± 0.6	5.4	1,780
0.5	12	0.3 ± 0.6	8.4	114,000
	24	0.7 ± 0.6	8.4	65,500
	48	1.3 ± 0.6	7.4	28,500
	72	1.3 ± 0.6	7.4	28,500
	96	0.7 ± 0.6	8.0	65,500
No treatment control		8.7 ± 0.6		1

Example 3 - Enhancing effect on the secondary production of antibody
against influenza HA vaccine:

　　　HA antigen prepared from influenza virus PR8 strain (A/Puerto
Rico/8/34, H1N1 type) that had been habituated to mouse was used as
an antigen for vaccination.　Attenuated cholera toxin was used as the

36

adjuvant. The residual toxic activity of attenuated cholera toxin used was 1/1780 to 1/114000 that of the original activity (according to Y-1 cell morphologic transformation test). Five Balb/c mice (6-weeks-old, females) were used for each group in the test. The mice
5 were anesthetized with sodium amobarbital, which was administered into the peritoneal cavity. 10 μl of PBS containing 1 μg vaccine antigen and 1 μg adjuvant was given dropwise to either nasal cavity of the mouse for intranasal immunization. Four weeks later, the secondary immunization was carried out in the same manner. Two weeks after the
10 secondary immunization, sera and nasal washes were collected from the mice.

The titer of anti-influenza virus antibody in the serum was determined based on the HI antibody titer. After bloodletting, the nasal washes were collected from the mice by perfusing the right and
15 left nasal cavities with 1 ml of PBS containing 0.1% bovine serum albumin (BSA). The quantities of anti-HA-IgA antibody in the nasal washes and anti-HA-IgG antibody in the sera were determined by enzyme immunoassay (ELISA). Prior to the assay for anti-HA-IgA, each well of EIA plate was treated with 50 μl of HA vaccine (5 μg/ml) suspended
20 in a coating buffer. The plate was allowed to stand still for the coating at room temperature for 2 hours. The plate was then washed with PBS containing Tween-20 (hereinafter abbreviated as PBS-Tween). Subsequently, each well was coated with 100 μl of PBS containing 1% BSA and 0.1% NaN₃ to prevent unspecific reactions. The plate was allowed
25 to stand still at 4°C overnight, and then washed with PBS-Tween. A 100-μl aliquot of adequately diluted nasal wash sample was added to each well. After several hours, the reaction solution was discarded and the well was washed with PBS-Tween. Subsequently, 100 μl of alkaline phosphatase-labeled goat anti-mouse IgA α chain-specific
30 antibody (or alkaline phosphatase-labeled goat anti-mouse IgG antibody) diluted with PBS containing 1% BSA and 0.1% NaN₃ was dispensed into the respective wells. The plate was allowed to stand still at room temperature for an hour and then washed. Finally, p-nitrophenyl phosphate (1 mg/ml; Sigma Co.) dissolved in 10% diethanol amine buffer
35 (pH 9.8) was added to each well for color development. The plate was allowed to stand still at room temperature for 20 to 30 minutes, and

then the coloring reaction was monitored at O.D. (405 nm) in a plate reader.

Figure 2 shows the influence of an influenza vaccine, comprising attenuated cholera toxin prepared in Example 2 as the adjuvant, on
5 the production of anti-influenza HA IgA antibody by the secondary response in the nasal wash. When the adjuvant-free vaccine was administered intranasally, the titer of anti-HA-IgA antibody was low. On the other hand, it was observed that the titer of anti-HA-IgA antibody in the nasal wash was markedly elevated in the group subjected to
10 primary and secondary inoculation of vaccine containing attenuated cholera toxin. The titer of antibody is comparable to that of the control in which the same dose of natural cholera toxin was used.

Figure 3 shows the influence on the production of anti-HA IgG antibody in the serum in the above-described test. The attenuated
15 toxin elevated 1/5 times the titer of HA antibody in the serum as compared with that with the adjuvant-free vaccine. However, the antibody titer was about half of that with natural cholera toxin used as a control. These results show that the attenuated cholera toxin is useful as the adjuvant for vaccination, for example, when the local immunity
20 is required to be enhanced.

Example 4 - Adjuvant activity of highly attenuated toxin:

A variety of attenuated cholera toxins, of which residual toxic activities were all at least one-two thousandth of that of the natural one, were used as the adjuvants. The same experiment as in Example
25 2 was repeated several times to assay the titer of anti-HA-IgA in the nasal wash and titer of IgG antibody in the blood of mice. The antibody titers determined in each assay were converted to relative values to those observed when the same amounts of natural cholera
30 toxin were use as positive controls. The relative value is indicated as the ordinate axis and the attenuation rate of the toxin (relative residual activity to the natural one) was indicated as the abscissa axis (Figure 4). It is obvious that the majority of attenuated toxins, of which residual toxic activities are at least one-two thousandth
35 that of the natural one (one-two thousandth $\fallingdotseq 4^{-5\ 46}$), exhibit high levels of antibody production-enhancing activity comparable to that of the

same amount of natural cholera toxin. In particular, it was verified that the attenuated toxins, of which residual toxic activities are reduced to at least one-two thousandth that of the natural one, show comparable or higher efficacy relative to that of original toxin in regard to the enhancement of mucous membrane IgA specific to the antigen by intranasal inoculation.

Example 5 - Use of dual adjuvant:

　　　A cholera toxin attenuated by the formalin treatment (having
10　a residual toxic activity of about 1/1048000 that of the natural one in Y-1 cell morphologic transformation test) and *E. coli* heat-labile toxin B subunit (untreated), which toxic activity had previously been confirmed to be weak, was used as the adjuvant together. This experiment was carried out in the same manner as in Example 3. The
15　cellular immune response to influenza virus was evaluated by the mouse foot swelling test. The result is shown in Figure 5. When 1 µg of *E. coli* heat-labile toxin B subunit and 0.1 µg or 0.01 µg of attenuated cholera toxin were used together, the adjuvant enhanced immune response evidently much higher than that exhibited with the same amount of
20　attenuated cholera toxin alone. The result shows that the dose of attenuated toxin can be further reduced by the method in which the attenuated toxin is used together with another adjuvant.

Example 6 - Preparation of various attenuated toxins and enhancing
25　effect thereof on the antibody production:

　　　Diphtheria toxin and pertussis toxin was prepared according to a method described by the Kitasato Institute for producing diphtheria vaccine and pertussis vaccine ("Textbook of techniques for vaccine production," Kitasato Institute, 1986). Each toxin was attenuated
30　in the same manner as shown in Example 2 and was adsorbed on aluminum gel and used in the subsequent experiments. In addition, commercial Staphylococcal α toxin (Sigma Co.) and enteritis vibrio thermostable toxin (Sigma Co.) were purchased and attenuated in the same manner as described in Example 2. The toxins were used without further
35　modification. A recombinant mutant LTR(7)K (lysine residue is substituted for arginine residue at amino acid position 7 from the

N terminus) of *E. coli* heat-labile toxin was prepared by a method described by Komase et al. (K. Komase et al., Vaccine 16, 248-254, 1998). Thus five types of attenuated toxins were obtained. The effect of enhancing immunity associated with influenza vaccine was tested
5 according to the same method as described in Example 3, except that the attenuated toxins prepared above were used at appropriate concentrations instead of the attenuated cholera toxin in Example 3. The result is shown in Table 3, where relative value of residual toxic activity of attenuated toxin (ratio of toxin attenuated versus
10 the natural one) is also indicated.

The result shows that both adjuvants of the invention, whether the natural toxin or the recombinant mutant toxin, exhibit activity of enhancing immunity associated with influenza vaccine administered by the intranasal vaccination route.

Table 3

Enhancing effect of various attenuated toxins on the antibody production

Attenuated Toxin	Ratio of attenuation $(1/4^n)$	Inoculation Volume (μg/mouse)		Antibody Produc- tion HI (2^n)
		Toxin	Influenza HA	
Staphylococ- cal α Toxin	6.7 9.1	0.5 0.5	2.0 2.0	9.0 8.6
Pertussis toxin	7.9 9.3	1.0 1.0	2.0 2.0	11.0 10.8
Diphtheria toxin	7.2	1.0	2.0	10.5
Recombinant of *E.coli* thermolabile toxin (LT-R7K)	5.8 9.3	5.0 5.0	2.0 2.0	10.9 11.8
Enteritis vibrio thermostable toxin	8.4	0.5	2.0	9.0
Chorea toxin (control)	0.0	1.0	2.0	11.2
No addition (control)		0.0	2.0	< 4

5 Example 7 - Pharmaceutical preparation containing pertussis-diphtheria-tetanus combined vaccine and attenuated cholera toxin (nasal drop):

Pertussis-diphtheria-tetanus combined vaccine was mixed with an attenuated cholera toxin (having a toxic activity of about 1/100000
10 as compared with that of the natural one) dissolved in PBS and sterilized by filtration. 20 μl of the solution contained the combined vaccine, of which amount corresponded to 50 μg of protein nitrogen, and 5μg of attenuated cholera toxin. A preservative (0.005% thimerosal) was added to the solution. The resulting mixture was dispensed into
15 appropriate containers, which was used as intranasal inoculum comprising pertussis-diphtheria-tetanus combined vaccine and attenuated cholera toxin. The preparation was stored at a temperature of less than 10°C in a cool and dark place.

Vaccine prepared as above containing pertussis-diphtheria-tetanus combined vaccine and attenuated toxin was inoculated to mice of each group. The same amount of the vaccine was further given to each mouse for supplementary inoculation after

5　4 weeks. After 2 weeks antibody production was evaluated. When vaccine alone was inoculated into mice, the titers (international unit) of anti-pertussis toxin antibody, anti-diphtheria toxin antibody, and anti-tetanus toxin antibody were 2.0 units or lower, 2.0 units or lower, and 1.5 units or lower, respectively. On the other hand,

10　the other group subjected to vaccination with attenuated toxin showed respective titers of 88.6, 61.8, and 75.5 units. The test result indicates that in the group subjected to vaccination with attenuated toxin, the amount of produced antibodies against the respective antigens are larger than those in mice inoculated with the adjuvant-free

15　vaccines.

Example 8 - Pharmaceutical preparation containing tetanus vaccine and attenuated cholera toxin (percutaneous inoculum):

　　　Tetanus toxoid was prepared as an antigen according to a method

20　described by Kitasato Institute for producing tetanus toxoid ("Textbook of techniques for vaccine production," Kitasato Institute, 1986). Cholera toxin and attenuated cholera toxin (having a toxic activity of about 1/2500 of the natural one) were prepared by the method described in Examples 1 and 2. The two were used as the adjuvants

25　in the test. A tetanus toxoid solution (400 Lf/ml) was mixed with a solution of either cholera toxin (4 mg/ml) or attenuated cholera toxin (4 mg/ml) at various volume ratios to give vaccine preparations (1 ml). After preparation, vaccine was immediately soaked into the cloth base (lint, about 2 cm x 2 cm) and then used for immunization.

30　Guinea pigs (5 in each group) were immunized by the following manner. The back of each guinea pig was shaved with a hair clipper, and then some epilation cream was spread on the back. After 30 minutes, the back of the guinea pig was washed. On the following day, cloth base on which the sample had been spread was patched on the back. The cloth

35　was fixed with an adhesive tape. The mice were bred in the usual manner. The cloth was changed with a fresh one once a week, four times in

total. The first blood collection was carried out on the fourth week after the first patch was given. Six weeks after the primary immunization, the fifth was patched (booster), and then one week after that (seven weeks after the primary immunization), the second blood
5   collection was performed.

A tetanus antibody assay kit "KAKETSUKEN" was used for assaying antibody titer in the blood. The assay result for the titer of antibody against tetanus toxin is shown in Table 4. After seven weeks, the antibody titer was found to be higher in the group subjected to vaccine inoculation with attenuated cholera toxin than that observed in the group subjected to vaccine inoculation without attenuated cholera toxin (control).

Table 4

Test group No.	Tetanus Toxoid (Lf/ cloth base)	Toxin (mg/cloth base)	Antibody titer in blood (international unit/mL)	
			After 4 weeks	After 7 weeks
1	100	Attenuated cholera toxin 1.0	3.6	12.3
2	100	Attenuated cholera toxin 0.1	2.6	9.2
3	100	Natural cholera toxin 1.0	4.1	25.6
4	100	Natural cholera toxin 0.1	2.6	14.3
5	100	No addition	2.0 or less	3.1

15

Example 9 - Hepatitis B vaccine-attenuated cholera toxin (injection):

Hepatitis B vaccine was combined with a fraction containing attenuated cholera toxin (having a toxic activity reduced to at least $1/10^6$ as compared with that of the natural one) that had been
20   dissolved in PBS and sterilized by filtration. 1 ml of the solution contained HBs antigen, of which amount corresponded to 40 μg of protein, and 10 μg of the attenuated cholera toxin. A preservative (0.01%

thimerosal) as well as a stabilizer (0.2% porcine gelatin) was added to the solution. The resulting mixture was dispensed into appropriate containers, and was used as the hepatitis B vaccine-attenuated cholera toxin injection. The preparation was stored at a temperature of less than 10°C in a cool and dark place.

5

The hepatitis B vaccine prepared above was inoculated into mice. The antibody production in the blood was evaluated after 3 weeks. The result of passive hemagglutination test showed that the antibody titer was $2^{3.6}$ units in mice subjected to the inoculation of adjuvant-free

10 vaccine and the titer was $2^{5.8}$ units in mice with attenuated cholera toxin.

Example 10 - Pharmaceutical preparation containing vaccine against Japanese encephalitis virus and attenuated cholera toxin (injection):

15 Japanese encephalitis vaccine was mixed with attenuated cholera toxin (having a toxic activity of at least 1/100000 that of the natural one) that had been dissolved in PBS and sterilized by filtration. 1 ml of the solution contained inactivated Japanese encephalitis virus particles corresponding to $10^{7.0}$ PFU and 2 μg of attenuated cholera

20 toxin. A stabilizer (0.2% porcine gelatin) was added to the solution. The resulting mixture was dispensed into appropriate containers, and was used as the Japanese encephalitis vaccine-attenuated cholera toxin injection. The preparation was stored at a temperature of less than 10°C in a cool and dark place.

25 The Japanese encephalitis vaccine prepared above was inoculated twice at a 1-week interval into mice. The antibody titer in the blood was evaluated. The titer of neutralizing antibody was $10^{1.8}$ units in mice subjected to the inoculation of the adjuvant-free vaccine, and the titer was $10^{2.9}$ units with attenuated cholera toxin.

30

Example 11 - Pharmaceutical preparation containing measles-rubella vaccine and attenuated cholera toxin (nasal drop):

Measles-rubella vaccine was mixed with attenuated cholera toxin (having a residual toxic activity of about 1/28500 that of the natural

35 one) dissolved in PBS and sterilized by filtration. 20 μl of the solution contained the respective vaccines of which amounts

corresponded to 7 μg virus particles, and 2.5 μg attenuated cholera toxin. A stabilizer (0.2% porcine gelatin, 0.1% sodium glutamate, 5% lactose) was added to the solution. The resulting mixture was dispensed into appropriate containers, and was used as the nasal drop

5　of measles-rubella vaccine-attenuated cholera toxin. The preparation was stored at a temperature of less than 10°C in a cool and dark place.

　　　The measles-rubella vaccine prepared above was inoculated twice at a 3-week interval into mice. Then the antibody production in the blood was evaluated. The test result showed that the ELISA antibody

10　titer was 0.18 for measles or 0.08 for rubella in mice subjected to the inoculation of the vaccine alone, and the titer was 0.34 for measles or 0.42 for rubella with attenuated cholera toxin-containing vaccine.

Example 12 - Pharmaceutical preparation containing measles-rubella
15　vaccine and attenuated pertussis toxin (nasal drop):

　　　The antibody titer in mouse blood was assayed according to the same method as described in Example 11 except that the attenuated pertussis toxin (the same as prepared in Example 6; having a toxic activity of about 1/100000 that of the natural one when observed by

20　CHO cell morphologic transformation test) was used instead of the attenuated cholera toxin used in Example 11. The ELISA antibody titer was 0.19 for measles or 0.070 for rubella in mice subjected to the inoculation of the adjuvant-free vaccine, and the titer was 0.32 for measles or 0.16 for rubella with attenuated pertussis toxin-containing

25　vaccine.

Example 13 - Pharmaceutical preparation containing rotavirus vaccine and attenuated recombinant *E. coli* heat-labile toxin (oral inoculum, nasal drop):

30　　　　Rotavirus vaccine was mixed with a fraction of attenuated recombinant LTR(7)K (having a toxic activity of about 1/260000 that of the natural one; the same as in Example 6) that had been dissolved in PBS and sterilized by filtration. 20 μl of the solution contained rotavirus vaccine, of which amount corresponded to 3.5 μg of virus

35　particles, as well as 10 μg attenuated recombinant LTR(7)K. A preservative (0.01% thimerosal) as well as a stabilizer (0.2% porcine

gelatin) was added to the solution. The solution was dispensed into appropriate containers, and was used as the oral inoculum or nasal drop of rotavirus vaccine-attenuated LT toxin. The preparation was stored at a temperature of less than 10°C in a cool and dark place.

5   The rotavirus vaccine prepared above was inoculated twice at a 3-week interval into mice. Then the antibody titer in the blood was evaluated. The test result showed that the ELISA antibody titer of the intranasal inoculation was 0.072 for the vaccine alone and was 0.40 for attenuated LT toxin-containing vaccine. The titer of

10 oral inoculation was 0.020 for adjuvant-free vaccine and was 0.19 for attenuated LT toxin-containing vaccine.

Industrial Applicability:

Examples described above demonstrate the following:

15 1. The adjuvant of the invention, comprising attenuated toxin, enhances the production of antibody against coexisting influenza vaccine antigen or the like.

2. Local antibody production, as well as the antibody production in the blood, is enhanced by the adjuvant of the invention when intranasally

20 administered together with vaccine antigen.

3. The adjuvant of the invention exhibits a comparable level of immuno-enhancing activity to that of the natural one at the same dosage, even when the residual toxic activity has been reduced to an undetectable level.

25 4. The antibody production can be enhanced synergistically in some cases when the adjuvant of the invention is used together with another conventional adjuvant. In other words, the dose of vaccine antigen can be reduced to decrease the incidence of side reaction by using the method in which the inventive adjuvant is used together with another

30 appropriate adjuvant.

Thus, vaccine preparations containing attenuated toxin in accordance with the present invention are useful as adjuvants with high safety. Further, vaccines using the adjuvant of the invention are not only highly safe but also are excellent vaccines having

35 sufficient activities of enhancing immunity even when used by non-injection vaccination route, such as intranasal, percutaneous

or oral inoculation, by which, with any conventional methods, sufficient immunological effects are not expectable. In addition, as seen in the Examples, the vaccines in accordance with the present invention are excellent in inducing local immunity, cellular immunity,

5   and such which were problems difficult to overcome with publicly known adjuvants. Moreover, it is also possible to utilize natural toxins as safe adjuvants according to the present invention. Thus, because of the simplicity, the invention is easily applicable to the vaccine as compared with approaches of generating mutant toxins.

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